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Thanks!

Lipid-Associated Sialoprotein in the Cerebrospinal Fluid

Association with Brain Malignancies

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BACKGROUND. Changes in the glycosylation process by tumor cells result in larger amounts of sialoproteins on their surface compared with normal cells. Sialoproteins then are released into the surrounding environment primarily by shedding or cell lysis. In the current study, the authors attempted to evaluate whether lipid-associated sialoprotein (LSP) in the cerebrospinal fluid (CSF) can distinguish patients with primary and metastatic brain tumors from those without brain tumors as well as determine response to treatment.

METHODS. CSF samples were obtained from a tissue bank. The concentration of LSP was determined after chloroform:methanol extraction followed by protein precipitation. One-way analysis of variance and Scheffe pairwise comparisons were used for statistical analysis.

RESULTS. The CSF of neurologically normal controls, patients with a normal leukocyte count ($\leq 5/\mu L$), and patients with various neurologic disorders or systemic tumors without central nervous system (CNS) malignancies contained similar levels of LSP. The CSF from patients with a normal leukocyte count and newly diagnosed primary or metastatic brain tumors contained on average 3.7-fold higher levels of LSP compared with CSF from patients without CNS tumors ($P = 0.0001$). The CSF from patients with brain tumors with progressive disease not responding to treatment contained high levels of LSP comparable to the levels found in newly diagnosed patients. The CSF from treatment-responsive patients contained decreased levels of LSP similar to that found in control patients.

CONCLUSIONS. The LSP in CSF may be a useful marker with which to determine the presence of intracranial malignancies and assess response to treatment. *Cancer* **2001;92:856–62.** © *2001 American Cancer Society.*

KEYWORDS: brain tumors, cerebrospinal fluid, central nervous system metastases, tumor marker, sialic acid.

Greater than 20,000 new cases of malignant primary brain tumors are diagnosed annually. By the time symptoms appear (due to cortical irritation, compression of nervous system structures, or obstruction of cerebrospinal fluid [CSF] flow) the majority of tumors have infiltrated or disseminated widely in the central nervous system (CNS), and surgery, cranial irradiation, and chemotherapy can provide only palliative benefit. Similarly, although advances in therapy have translated into longer survival for patients with extraneural disease, these advances have, ironically, increased the number of patients who develop CNS metastases.[1–4] CNS metastases also are an important site of disease recurrence after high-dose chemotherapy with bone marrow or stem cell transplantation. Greater than 150,000 patients are diagnosed with CNS metastases each year.[5] Again, the diagnosis generally occurs after the onset of neurologic manifes-

TABLE 1
LSP Concentration in the CSF of the Various Patient Groups

Category	Diagnosis	No. of patients	Age ± SD	[LSP] mg/L (mean ± SD)	ln [LSP] (Mean ± SD)
NNC	Normal neurologic controls	12	35 ± 21	1.38 ± 0.75	0.21 ± 0.47
NC	Neurologic controls	14	49 ± 22	1.25 ± 0.92	0.15 ± 0.71
MEN	Microbial meningitis	6	59 ± 11	6.78 ± 3.22	1.83 ± 0.45
STNM	Systemic tumors with no metastasis to the brain	16	59 ± 15	1.48 ± 0.95	0.18 ± 0.70
PBT	Primary brain tumor, newly diagnosed	10	54 ± 20	6.03 ± 4.76	1.53 ± 0.78
MBT	Metastatic brain tumor, newly diagnosed	12	68 ± 14	4.72 ± 2.49	1.44 ± 0.49
PD	Progressive disease	22	52 ± 18	7.18 ± 4.64	1.75 ± 0.63
SD	Stable disease	8	53 ± 15	2.10 ± 0.75	0.67 ± 0.41
PR	Partial response	12	55 ± 23	2.27 ± 0.79	0.76 ± 0.40
CR	Complete response	5	45 ± 15	1.23 ± 0.89	−0.02 ± 0.76

LSP: lipid-associated sialoprotein; CSF: cerebrospinal fluid; SD: standard deviation.

tations and heralds a rapidly fatal course for the vast majority of patients. Modern neuroimaging techniques such as magnetic resonance imaging (MRI), MRI spectroscopy (MRI SPECT), and positron emission tomography (PET) have facilitated the early diagnosis and subsequent monitoring of patients with CNS malignancies, but these studies are expensive and are limited by false-negative and false-positive findings in a number of commonplace situations including CNS infections, postoperative changes, and radiation-related and chemotherapy-related brain injury. To our knowledge, no reliable and efficient screening test that can detect brain metastases at a subclinical stage to initiate treatment in patients at high risk is currently available. An assay that can detect tumor recurrence, predict response to therapy, and distinguish between persistent or recurrent tumor and treatment-related changes in patients with primary brain tumors also is currently lacking.

A number of compounds currently are used as tumor serologic markers in patients with tumors outside the CNS. These markers primarily are tumor antigens, and the majority are useful for disease monitoring. Familiar examples include squamous cell carcinoma (SCC) antigen, prostate specific antigen (PSA), carcinoembryonic antigen (CEA), CA 125, and CA 15-3. More recently, growth factors (e.g., basic fibroblast growth factor) and protooncogene products (e.g., c-*erb* B-2) also have been investigated.[6–12]

The CSF may provide another informative and relatively accessible source of biologic markers in patients with CNS tumors. Standard laboratory analyses such as glucose and protein have no consistent correlation with the presence or disease activity of CNS tumors, and in patients with carcinomatous meningitis, CSF cytology provides only a 50–70% sensitivity.[12] Although conventional tumor antigen markers in the CSF are of modest benefit in helping to assess response to therapy in patients with carcinomatous meningitis,[13–17] again, no usefulness has been shown in patients with brain metastases or primary brain tumors.

Recently, a few groups, including our own,[13–18] have reported novel biologic markers with high enough specificities and sensitivities to be useful for the diagnosis and monitoring of both carcinomatous meningitis and primary and metastatic brain tumors. The current study describes our experience with a promising CSF marker for CNS malignancy, lipid-associated sialoprotein (LSP).

MATERIALS AND METHODS

CSF Sampling

The protocol for CSF collection banking and subsequent investigational use was approved by each institution's investigational review committee and informed consent was obtained from all patients. All CSF specimens were transferred to polyethylene tubes and were stored in a −70 °C freezer until being thawed for analysis. Cell count and protein and glucose analysis data were available for all CSF specimens. In addition, CSF samples from patients with cancer were examined cytologically for malignant cells at the time the CSF was obtained.

Neurologically normal controls

Twelve CSF samples from neurologically normal patients constituted 1 control group[19] (Table 1). In three of these patients, CSF was obtained by lumbar puncture as part of spinal anesthesia (one patient undergoing caesarean section, one patient undergoing tubal ligation, and one patient undergoing a urologic procedure). In four febrile patients CSF was obtained to rule out meningitis. The remaining five patients presented with neurologic symptoms (three with head-

ache and two with memory loss) but had normal neurologic examinations, CSF studies, and head MRIs (Table 1). All CSF samples had normal levels of protein (15–45 μg/mL), glucose (40–80 mg/mL), and leukocytes (≤ 5/μL).

Neurologic controls

Twenty CSF samples from patients with established neurologic diagnoses constituted the neurologic control group and have been described earlier.[19] These patients included one patient with Alzheimer disease, one patient with multiple sclerosis, one patient with ischemic infarction, one patient with intracerebral hemorrhage, one patient with subarachnoid hemorrhage, one patient with epidural hematoma, one patient with lumbar radiculopathy, one patient with hydrocephalus, two premature infants, one patient with Lyme neuroborreliosis, one patient with pseudotumor cerebri, one patient with systemic lupus erythomatosus, one patient with neurologic complications after renal transplantation, one patient with a vascular malformation, and six patients with bacterial, viral, or fungal meningitis. All the CSF samples, except the CSF samples from patients with meningitis, contained a physiologic leukocyte count (≤ 5/μL).

Samples from patients with cancer

A total of 109 CSF samples from patients with cancer constituted the study group. These included 10 patients with newly diagnosed primary brain tumors (2 with anaplastic astrocytoma, 4 with glioblastoma multiforme, 1 with ependymoma, 1 with neurocytoma, 1 with oligodendroglioma, and 1 with primary CNS lymphoma) and 12 patients with systemic tumors (4 with breast carcinoma, 2 with melanoma, 2 with nonsmall cell lung carcinoma, 1 with small cell lung carcinoma, 1 with renal carcinoma, 1 with myeloma, and 1 with prostate carcinoma) and newly diagnosed brain metastases who had not received treatment for their intracranial tumor. Forty-seven samples were from patients with brain tumors who were receiving treatment and 16 samples were from patients with systemic cancer without brain metastases. Twenty-four samples were from 6 patients with neoplastic meningitis who underwent serial CSF sampling during the course of their disease. Again, all CSF samples contained leukocyte counts ≤ 5/μL.

Determination of Lipid-Associated Sialoprotein

The method we use takes advantage of the fact that only a small percentage of sialic acid in the circulation is lipid-associated.[20] We measured the lipid-associated sialoprotein (LSP) levels in the CSF from patients with CNS malignancies and other neurologic diseases as follows: half of a milliliter of CSF is dried under a nitrogen stream and the dry residue is dissolved in water. The aqueous solution is extracted with a double volume of a chloroform:methanol mixture. The upper phase is removed and any proteins in it are precipitated with phosphotungstic acid. The precipitated proteins are dissolved by boiling in 0.2% resorcinol in 30% hydrochloric acid and 0.25 mM copper sulfate. The concentration of LSP is determined by the absorption at 580 nanometers compared with a standard curve generated using known amounts of sialic acid.

Statistical Analysis

One-way analysis of variance (ANOVA) or Student *t* tests were used in the logarithmic scale for comparing the LSP levels between the different patient groups. A logarithmic transformation of LSP levels was necessary to satisfy the normality assumption. When more than two groups were compared, post hoc pairwise comparisons were performed according to Scheffe.[21] Kruskal–Wallis analysis on the original data scale led to the same conclusions.[22] All reported *P* values were two-sided and results were considered significant at the 0.05 significance level. Cutoff values for a positive test were determined using a receiver operating characteristics (ROC) curve. The SAS statistical package was used for the statistical analysis.[23]

RESULTS

LSP in the CSF of Control Patients and Patients with Newly Diagnosed CNS Malignancies

The concentration of LSP in the CSF from the neurologically normal control patients was 1.38 ± 0.75 mg/L. Similarly the CSF samples from 14 patients with various nonmalignant neurologic diseases contained 1.45 ± 0.92 mg/L of LSP. CSF from the 6 patients with bacterial, viral, or fungal meningitis had elevated leukocyte counts (14–885 leukocytes/μL, with a mean of 207 and a mean of 75). The LSP level in this group of patients was elevated (6.78 ± 3.22 mg/L) compared with neurologic controls (Table 1) (Fig. 1a).

The CSF from patients with newly diagnosed primary and metastatic brain tumors who had not received any treatment for their CNS malignancy contained 6.03 ± 4.76 mg/L and 4.72 ± 2.49 mg/L LSP, respectively (Table 1). This was significant compared with the LSP concentration found in the CSF of patients without any neurologic diseases and patients with established neurologic diseases (ANOVA, $P = 0.0001$). Because patients with systemic cancer are at risk to develop brain metastases, we quantitated the LSP concentration in the CSF of patients with systemic tumors without brain metastasis. Samples from 16 patients with tumors including lung carcinoma (8 pa-

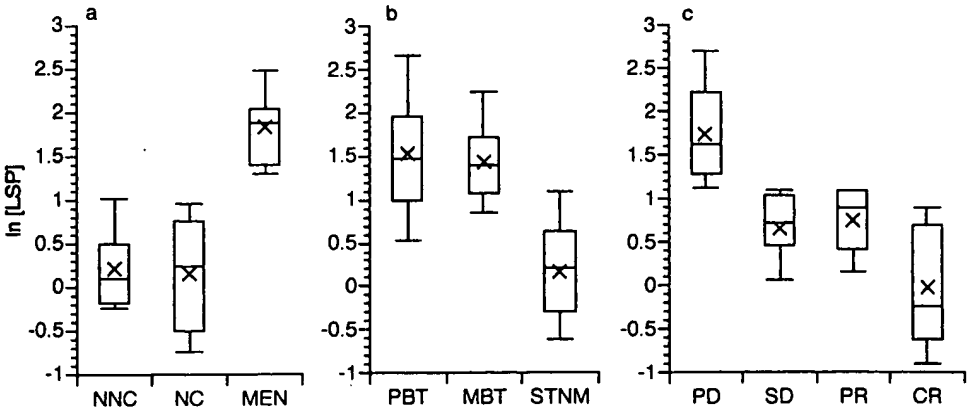

FIGURE 1. Lipid-associated sialoprotein (LSP) in the cerebrospinal fluid (CSF) of patients with and without malignancies. Box plot showing in a logarithmic scale the 75th, 50th, and 25th percentile. Whiskers point to the 90th and 10th percentile. X indicates the mean. a) LSP in the CSF of patients without malignancies. b) LSP in the CSF of patients with malignancies. c) LSP in the CSF of patients with central nervous system malignancies receiving treatment. NNC: normal neurologic controls; NC: neurologic controls; MEN: patients with meningitis and a leukocyte count > 5/μL; PBT: patients with newly diagnosed primary brain tumors; MBT: patients with newly diagnosed metastatic brain tumors; STNM: patients with systemic tumors but no metastasis to the brain; PD: patients with progressive disease not responding to treatment; SD: patients with stable disease; PR: patients with a partial response to treatment; CR: patients with a complete response to treatment and no residual tumor.

tients), lymphoproliferative disorders (5 patients), colon carcinoma (two patients), and breast carcinoma (one patient) were studied. They contained 1.48 ± 0.95 mg/L of LSP, which was similar to the amount found in neurologically normal controls and individuals with neurologic disease and which was significantly different from the level in patients with systemic tumors metastatic to the brain (ANOVA, $P = 0.0001$) (Table 1) (Fig. 1b).

An ROC curve of LSP levels as an indicator of metastatic brain tumors in patients with systemic tumors with and without brain tumors was prepared (Figure 2). Individuals with systemic tumors comprised the risk group of interest for the development of brain metastases. With 2.5 mg/L as a cutoff value, we obtained a 91.7% sensitivity and 81.2% specificity. Using the incidence of tumor metastases to the brain (150,000) and the cancer incidence in the U.S. population for 1999 (1,221,800),[24] we obtained a positive predictive value of 0.348 and a negative predictive value of 0.989 for the assay. When all values obtained from CSF samples from patients with physiologic leukocyte counts (≤ 5/μL) without CNS malignancies were combined, the average LSP concentration in the CSF was 1.44 ± 0.87 mg/L. If we use the cutoff value of 2.5 mg/L for a positive test in these groups, the assay has an 88% sensitivity for the groups of patients with primary and metastatic brain tumors and 82% specificity for the groups of patients including neurologically normal controls, neurologic controls, and patients with systemic tumors but without CNS involvement.

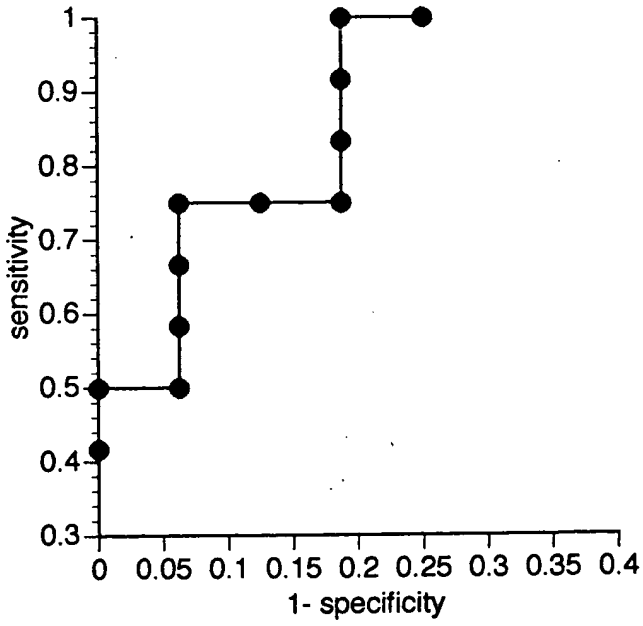

FIGURE 2. Receiver operating characteristic curve of lipid-associated sialoprotein levels as an indicator of metastatic brain tumors in patients with systemic tumors with and without brain metastases.

LSP in the CSF as an Indicator of Tumor Behavior

To explore whether the LSP concentration in the CSF reflects tumor activity and response to treatment, we classified patients with primary and metastatic brain tumors as complete responders (CR), partial responders (PR), stable disease (SD), or progressive disease (PD) at the time of CSF sampling according to the criteria of Macdonald et al.[25] The CSF from 22 patients with PD

TABLE 2
Characterization of Patients from Whom Serial CSF Samples were Obtained

Patient	Age (yrs)	Gender	Tumor type	Treatment for neoplastic meningitis
1	34	M	Anaplastic astrocytoma	Intrathecal methotrexate
2	34	M	Glioblastoma multiforme	Intrathecal thiotepa
3	23	M	Glioblastoma multiforme	Intrathecal thiotepa
4	86	F	Primary CNS lymphoma	High-dose intravenous methotrexate
5	38	M	Primary CNS lymphoma	High-dose intravenous methotrexate
6	61	F	Primary CNS lymphoma	Intrathecal sustained-release Ara-C

CSF: cerebrospinal fluid; M: male; F: female; CNS: central nervous system; Ara-C: cytarabine.

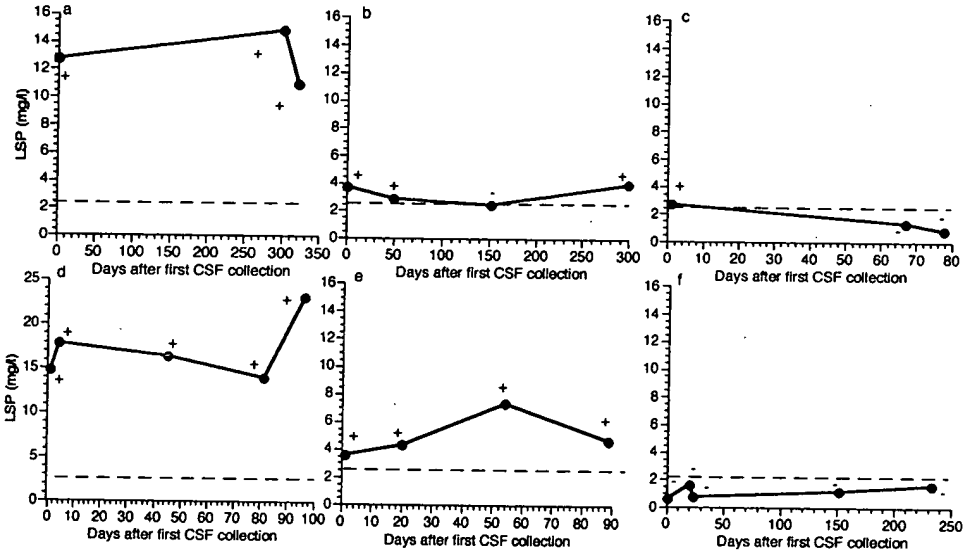

FIGURE 3. Longitudinal analysis of lipid-associated sialoprotein (LSP) in the cerebrospinal fluid (CSF) of patients receiving treatment. (a–f) CSF samples from 6 patients with central nervous system with malignancies collected at various time points during treatment were analyzed for the presence of tumor cells and the levels of LSP. (+) indicates of positive CSF cytology and (−) indicates negative CSF cytology. The dashed line defines the normal LSP threshold level of 2.5 mg/L.

contained 7.18 ± 4.64 mg/L LSP, which is similar to the levels of patients with newly diagnosed brain tumors (Table 1) (Fig. 1c). The LSP levels in the CSF of patients in the other response categories were substantially lower than the levels found in the CSF from patients with PD (ANOVA, $P = 0.0001$). The CSF from 8 patients with SD contained 2.10 ± 0.75 mg/L LSP. The CSF from 12 patients with PRs contained 2.27 ± 0.79 mg/L LSP and the CSF from 5 patients with CRs contained 1.23 ± 0.89 mg/L. These results suggest that the level of LSP in the CSF reflects the tumor activity and is a marker for tumor progression and response to treatment.

LSP in Longitudinally Collected CSF Samples from Patients with Neoplastic Meningitis

Using a second strategy to determine whether the CSF LSP concentration is correlated with tumor response to treatment, we measured the LSP concentration in CSF specimens collected serially from individual patients (Table 2). In six patients with cytologically documented neoplastic meningitis, serial CSF sampling and LSP determinations were performed during the course of therapy. Figure 3 illustrates the correlation between the CSF cytology and LSP concentrations over time. As the tumors responded to the treatment and the cytology became negative there was a reduction of the LSP concentration below the 2.5 mg/L threshold. If there was no improvement and the cytology was maintained positive for malignant cells, the LSP concentration remained consistently above the 2.5 mg/L level (Fig. 3).

DISCUSSION

To our knowledge, there are few studies published to date that deal specifically with brain tumor markers.[15,17,18,26–30] The majority of brain tumor markers reflect chromosomal abnormalities such as p53 gene mutations, q18 translocations, epidermal growth factor, and fibroblast growth factor expression. However, these markers are evaluable when biopsy specimens are obtained and the tumor itself is analyzed. Although these markers may have diagnostic and prognostic value they cannot be evaluated repeatedly at clinically relevant intervals, and cannot be depended on to assess response to ongoing therapy to answer new diagnostic questions (e.g., to distinguish between

recurrent tumor and treatment-induced differences) or to monitor patients with clinically SD. The CSF may be a source of biologic indicators that can provide information regarding the presence of a tumor in the CNS and its status. Any material produced by the tumor or the surrounding cells in response to the tumor potentially can diffuse or be secreted into the CSF. Standard laboratory analyses that measure CSF protein and glucose appear to have no correlation with the presence of CNS malignancies.[31] CSF cytology is useful in cases of neoplastic meningitis when malignant cells may be detected in the CSF. Several recent studies discuss the value of CSF cytology for the diagnosis of leptomeningeal carcinomatosis, with a sensitivity ranging from 50–70%.[32] In the current study, we investigated whether a specific fraction of sialic acid can be used as a marker for brain tumors.

Changes in the metabolism of sialic acid by tumor cells are characterized by aberrant glycosylation processes by tumor cells resulting in larger amounts of sialic acid on their surface compared with normal cells.[33,34] In 1977 Kloppel et al., for what to our knowledge was the first time, used serum lipid-associated sialic (LASA) acid as a marker for cancer.[35] Greater than 30 studies have investigated whether total and lipid-soluble sialic acid is a useful tumor marker.[36] In comparison studies, LASA has been found consistently to be more reliable than other markers including SCC antigen in patients with head and neck carcinoma;[7] PSA in patients with localized and metastatic prostate carcinoma;[6] CEA in patients with breast carcinoma, lung carcinoma, leukemia, lymphoma, Hodgkin lymphoma, and melanoma;[37] and CEA, tissue polypeptide antigen and CA 19-9 in patients with laryngeal carcinoma.[36] Only in the case of ovarian malignancies do the results conflict with different laboratories reporting various sensitivity and specificity values.[38–40]

In the current study, we used a method to determine the level of a specific fraction of sialic acid that is both lipid-soluble and protein-bound (LSP). We found that the CSF from patients with newly diagnosed brain tumors contained significantly higher amounts of LSP than the CSF from three pertinent control groups: neurologically normal patients, patients with established neurologic diseases, and patients with systemic cancer without CNS involvement ($P = 0.0001$ for all comparisons). Using an ROC-determined cutoff value of 2.5 mg/L, the CSF LSP concentration can distinguish between cancer patients with and without CNS tumors with a very high negative predictive value (0.989) and lower (but respectable) positive predictive value (0.348) in the general cancer population, where the incidence of CNS involvement is relatively low (approximately 1.4%). Thus measurement of the CSF LSP concentration may be a very useful screening test for certain populations of cancer patients such as those preparing for high-dose chemotherapy with bone marrow or stem cell support (in which CNS involvement generally is considered an exclusion to such therapy), or in patients with a relatively high incidence of CNS cancer such as patients with malignant melanoma, small cell lung carcinoma, recurrent breast carcinoma, or nonsmall cell lung carcinoma, or patients with previously diagnosed CNS disease that has responded completely to treatment (e.g., patients with solitary brain metastases). Because this assay also appears to distinguish treatment-responsive patients with CNS malignancies from patients whose tumors are not responding, the measurement of the CSF LSP also may be used as a surrogate marker in patients receiving treatment, particularly when the neuroradiographic studies are equivocal (e.g., patients with brain metastases after stereotactic radiosurgery, or patients with primary brain tumors after cranial irradiation), in patients whose clinical and radiographic studies are conflicting, and in patients with SD after the completion of therapy. In our small series, the CSF LSP concentration also was found to correlate perfectly with CSF cytology in patients with cytologically documented neoplastic meningitis. The suggested threshold of 2.5 mg/L should be viewed with caution because several samples were minimally different from the cutoff value. At the same time, a competently performed lumbar puncture is only mildly uncomfortable; is universally accessible; is inexpensive compared with biopsy, MRI, MR SPECT, or PET; and, with few and well defined exceptions, is safe even in patients with CNS malignancies.

The LSP assay has limitations. CNS inflammation may reduce the value of the assay because leukocytes can produce large amounts of LSP. However, radiation, chemotherapy, and CNS cancer are not typically associated with an increased leukocyte (none of our 109 CSF samples in the current series) and such cases will be easily identified because CSF samples are always tested for the presence of an elevated leukocyte count.[31] LSP also is a histologically nonspecific tumor marker. This is not a disadvantage because the goal of the CSF LSP assay is not to determine the type of tumor but rather whether there is a tumor in the CNS in patients with known cancer who are at risk for CNS metastases, or in patients with a known CNS malignancy at some stage in their treatment. We currently are performing studies to compare the LSP assay with MRI and CSF cytology in patients without a known CNS malignancy and studies to evaluate CSF LSP as marker for treatment response in patients with a known CNS malignancy. We feel that this assay has the potential to be used as a diagnostic and prognostic tool.

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